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Significance of Hemolytic Activity of Some Radiation-Resistant Micrococci in Food†

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Micrococci resistant to 1 Mrad of gamma radiation were isolated from irradiated chicken. Three isolates were hemolytic on blood agar plates and were selected for further study. Two other radiation-resistant micrococci, *Micrococcus radiodurans* and *Micrococcus radiophilus*, were included in the study because there is only a very limited amount of information regarding hemolytic activity of these organisms and their potential role of public health importance. Tests to determine hemolytic patterns, hemolytic activity of extracellular substances, leukocytic activity, presence of enzymes commonly associated with pathogenicity (coagulase, deoxyribonuclease, phosphatase), and pathogenicity for laboratory animals all suggested that the organisms would not be of public health significance.

Hemolytic radiation-resistant micrococci were isolated from chickens during the course of a study of naturally occurring radiation-resistant asporogenous bacteria in meat (5, 7). These bacteria were obtained from samples which had been subjected to 1 Mrad of gamma radiation. Although hemolysis in itself is not necessarily a criterion of pathogenicity, there is reason for some concern since the majority of pathogenic *Micrococcaceae* are hemolytic (2).

Asporogenous radiation-resistant bacteria may constitute a high proportion of the total number of bacteria found in irradiated fresh meat (6), with the proportion increasing directly with radiation. Thus, food treated with sterilization doses of radiation may carry a high percentage of radiation-resistant bacteria. If any of these bacteria are of public health significance, their presence in such food would be a concern because information on their public health significance is limited. The significance of these hemolytic micrococci as potential pathogens was determined by studying cytotoxic activity, cellular enzymes, and pathogenicity in animals.

MATERIALS AND METHODS

Bacteria and culture conditions. Three isolates of hemolytic radiation-resistant micrococci, designated C-3, C-4, and C-7, were recovered after 1 Mrad of gamma radiation of vacuum-packed frozen, ground skin, muscle, and cartilage of chicken wings. Characterization of these bacteria, including their patterns of radiation resistance, has been described (8). *Micrococ-*

cus radiodurans (ATCC 13939) and *Micrococcus radiophilus* (N. F. Lewis, Bhabha Atomic Research Center, Bombay, India) were included in some tests for comparative purposes. The bacteria were propagated on plate count agar (Difco) or tryptic soy agar (TSA; Difco) slants, although *M. radiophilus* does not grow luxuriantly on plate count agar. To obtain high concentrations of cells, test cultures were inoculated into flasks of tryptic soy broth (Difco) or tryptose phosphate broth and grown to heavy turbidity in a 32°C incubator shaker.

Hemolysis of erythrocytes in agar. TSA and heart infusion (Difco) blood agar plates were prepared using 5% fresh defibrinated sheep, rabbit, and human blood. Plates were inoculated with the organisms by the streak and stab method, incubated at 32°C, and observed daily for 10 days. Plates were examined visually under various light conditions and with a dissecting microscope with 7 to 30× magnification.

Tests for hemolysins in culture filtrates. Erlenmeyer flasks containing tryptose broth were inoculated with C-3, C-4, and C-7 and incubated at 32°C in a 20% CO₂ atmosphere, since incubation in 20% CO₂ has been reported to enhance hemolysin production (2). *Staphylococcus aureus* was included as the positive control. C-3, C-4, and C-7 did not grow well in CO₂, and, therefore, subsequent cultures were grown in an incubator shaker. When cultures contained approximately 10⁸ organisms per ml, they were centrifuged for 15 min at 1,500 × g and the supernatant fluids were filtered through a membrane prefilter (Millipore Corp.).

Twofold serial dilutions of the supernatants were prepared using both 0.85% NaCl and phosphate-buffered saline as diluents, with the first tube in each series being undiluted. Sheep and rabbit erythrocytes, previously washed three times in NaCl, were added separately to tubes of diluted supernatants to give a final concentration of 0.3%. Tubes were incubated for 1 h in a 37°C water bath, observed for hemolysis of

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erythrocytes, and then placed at 5°C for 18 h and again observed.

Tests for leucocidin. Flasks of tryptic soy broth were inoculated with C-3, C-4, C-7, *M. radiodurans*, and *M. radiophilus*, incubated for 48 h at 32°C, and centrifuged at $6,000 \times g$ for 20 min. Supernatant fluids were used for leucocidin assays. Samples of rabbit blood and of human blood, collected in anticoagulant, were centrifuged in 5-mm-diameter tubes. The leukocytes were withdrawn and diluted in 0.85% NaCl, and the total leukocyte count was determined with a hemacytometer. Equal volumes of leukocytes and each sample of supernatant culture fluid were mixed and incubated for 1 h at 37°C, and total leukocyte counts were again determined. As a positive control, a culture of *S. aureus* was treated in the same manner as test samples. Tubes, consisting of leukocytes incubated with tryptic soy broth instead of culture fluids, were used as negative controls.

To determine numbers of viable leukocytes, trypan blue dye was added to a portion of the leukocytes before incubation with culture fluids and to a comparable sample after incubation. These samples were examined microscopically to determine the ability of cells to exclude trypan blue as an indicator of viable cells.

Injection of animals. Five- to six-week-old mice were injected intraperitoneally with 0.2 ml of the supernatant of the culture fluids, and adult rabbits were injected intradermally with 0.3 ml. Supernatant culture fluid of *S. aureus* was used as a positive control.

Cultures of C-3, C-4, and C-7 were inoculated onto agar slants, incubated for 48 h, and harvested with

0.85% NaCl. Smooth suspensions containing more than 6×10^9 colony-forming units per ml were obtained. A 0.2-ml volume of each suspension was injected intraperitoneally into weanling (2- to 3-week-old) and adult (5- to 6-week-old) mice.

Tests for coagulase, phosphatase, and DNase. Coagulase reactions were determined with the slide technique for bound coagulase, the tube technique for bound coagulase, and the tube technique for free coagulase using reconstituted desiccated rabbit plasma (Difco). Tests for phosphatase were determined by the procedures described by White and Pickett (9). Deoxyribonuclease (DNase) test agar (Difco) was used to test for presence of DNase (3). *S. aureus* was used as a positive control with all tests.

RESULTS

Hemolysis of erythrocytes in agar. Hemolysis of erythrocytes and/or discoloration of media was apparent after 48 h of incubation. The only change noted after 5 days was that, after approximately 1 week, often the entire plate became brown. Therefore, the results in Table 1 are for observations at 2 and 5 days only. A typical example of the hemolysis produced by these micrococci is shown in Fig. 1. When observed without magnification, it was not possible to determine if hemolysis was complete or partial. When plates were observed under low magnification (7 to 30 \times), it became apparent that hemolysis of erythrocytes was only partial (Fig.

TABLE 1. Hemolytic reactions on blood agar plates^a

Micrococcus	Rabbit erythrocytes		Sheep erythrocytes		Human erythrocytes	
	HIA ^b	TSA	HIA	TSA	HIA	TSA
C-3						
2 days	gr,H,P,D	gr,D	gr	gr	—	P,D
5 days	gr,H,P,D	gr,br,P,D,H, S	gr,br	gr,B,P,D	slight gr,H,P,D, S,H	gr,H,P,D,S,H
C-4						
2 days	—	—	gr	—	P,D	P,D
5 days	gr,H,I,P,D, H,S	P,D,H,S	slight gr	gr,P,D,H,S	gr,H,P,D,S, H	gr,H,P,D only
C-7						
2 days	gr,H,P,D	gr,D	gr,P,D	gr	gr,H,P,D	—
5 days	gr,br,P,H,S, D	gr,br,P,D,S, H,I	gr,br,P,D,H, S	gr,P,D,S,H,I	gr,H,P,D,S, H	gr,H,P,D,S,H
<i>M. radiodurans</i>						
2 days	gr,H,P,D	gr,P,D	gr,P,D	gr,P,D	gr,H,P,D	—
5 days	gr,H,P,D,H, S	gr,C,D,S,H,I	gr,P,D	gr,P,D,S,H,I	gr,H,P,D,S, H	gr,H,P,D,S,H
<i>M. radiophilus</i>						
2 days	gr,D,P,D	gr,P,D	gr,P,D	gr,P,D	gr,H	P,D
5 days	gr,H,P,D	gr,P,D,S,H,I	gr,P,D,H,S	gr,P,D,S,H,F	gr,H,P,D,S, H	gr,D only, P,H,S or D

^a Legend: —, no change; discoloration of medium around colonies—gr (green), or br (brown); P, partial lysis of erythrocytes; C, complete lysis of erythrocytes; S, surface colonies; D, deep colonies, i.e., those growing under the agar; H, reaction occurring where growth is heavy; I, reaction occurring around individual colonies.

^b HIA, Heart infusion agar.

2A). The only zone of complete hemolysis was produced by *M. radiodurans* incubated for 5 days on TSA containing rabbit erythrocytes (Fig. 2B). Zones of hemolysis were never observed to extend more than 3 mm beyond the edge of the colony.

Tests for hemolysins in culture filtrates. No hemolysis of erythrocytes was observed in any of the tubes prepared with supernatant of culture fluid from C-3, C-4, or C-7 at 1 or 18 h. The positive control with *S. aureus* was hemolytic at 1 h, and the same titer was observed after 18 h.

Tests for leucocidin. There was no decrease in total number of leukocytes incubated with supernatants of culture fluids as compared with negative control samples. After incubation with culture fluid supernatant of *S. aureus*, the number of leukocytes was reduced by 78%. Also, there was no increase in the number of leukocytes taking up trypan blue dye, which demonstrated that there was no reduction in viability of leukocytes during incubation with the supernatant of culture fluids.

Reaction of animals. No reaction was detected in any of the mice during the 4 weeks of observation after injection with supernatants of culture fluids. No reaction was produced in the rabbits by C-3, C-4, or C-7. However, in the

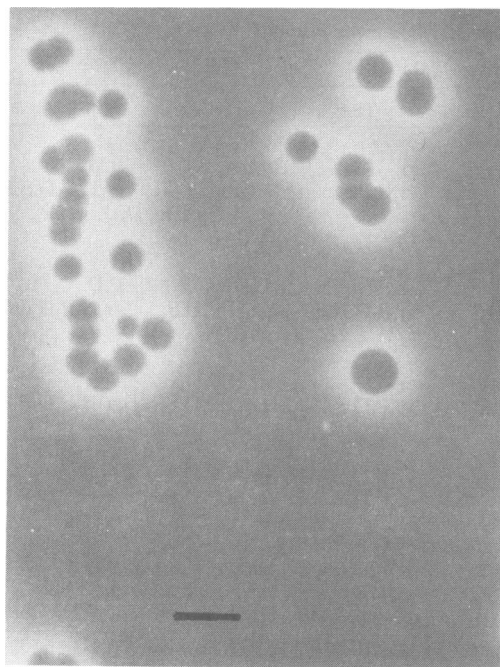


FIG. 1. Hemolytic reaction produced by isolate C-7 on TSA with sheep erythrocytes. Bar = 5 mm.

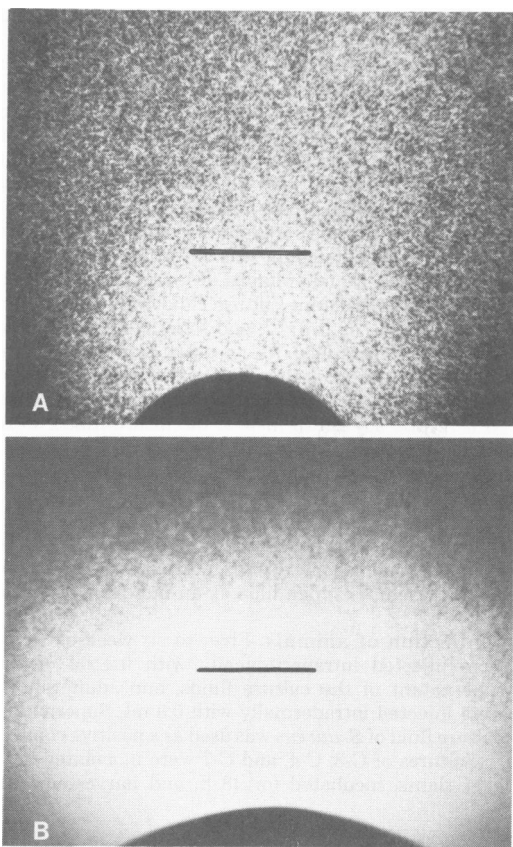


FIG. 2. Enlarged photographs of edge of colonies and erythrocytes surrounding colonies. TSA and rabbit erythrocytes. (A) Partial hemolysis produced by isolate C-7. (B) Complete hemolysis produced by *M. radiodurans*. Bar = 0.5 μ m.

control of *S. aureus*, a distinct and long-lasting erythema, 10 to 12 mm in diameter surrounding the site of injection, developed between 24 and 48 h in rabbits inoculated with the supernatant fluid.

None of the mice injected intraperitoneally with viable C-3, C-4, or C-7 displayed any signs of abnormality during the 4 weeks of observation.

Tests for coagulase, phosphatase, and DNase. Tests for coagulase, phosphatase, and DNase were negative with C-3, C-4, C-7, *M. radiodurans*, and *M. radiophilus*. All of these tests were positive when using *S. aureus*.

DISCUSSION

When these micrococci were first isolated and observed to be hemolytic on blood agar, there was concern about their public health significance. Upon initial examination, the exact type

of hemolysis was not evident, since the zone of hemolysis was narrow and frequently there was greening and browning of the media (Table 1). Detailed studies of these micrococci and their cellular enzymes were therefore utilized as indicators of a possible role for these bacteria in public health.

In our studies, erythrocytes from various species of animals were used in both the plate and tube techniques to identify types of hemolysins. Results (Table 1) demonstrated that all of the micrococci studied, with the exception of *M. radiodurans*, produced only narrow zones of partial hemolysis under all of the conditions utilized. *M. radiodurans* produced a narrow zone of complete hemolysis only on TSA containing rabbit erythrocytes. Anderson et al. (1) previously reported that *M. radiodurans* showed a weak beta-type hemolysis, after 1 week of incubation, but did not specify the type of media or erythrocytes used. Thus, it is apparent that detailed examination is necessary to differentiate between partial and complete hemolysis. Likewise, it is necessary to utilize more than one medium to generalize that an organism is hemolytic. Patterns of hemolysis observed in our studies differed from those produced by known pathogenic species of *Micrococcaceae*. In addition, the negative results of in vitro tests for lytic activity of culture filtrates on erythrocytes and leukocytes further indicated that the bacteria would not be of public health significance.

When these culture filtrates, as well as viable micrococci, were used in animal studies, they provided further results of negative indications of pathogenicity. Lewis (4) had previously concluded that *M. radiodurans* and *M. radiophilus* were nonpathogenic because filtrates and sonicated cell suspensions were not detrimental to mice. Our broader studies, which included using

viable cells as well as culture filtrates and rabbits and mice of different ages, demonstrated that none of the micrococci studied was pathogenic to laboratory animals. Negative results of coagulase, phosphatase, and DNase further indicated that these micrococci were not pathogenic.

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